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Original Article

Effect of Platelet Releasate on uPA and uPAR Gene Expression in Mesenchymal Stem Cells

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Abstract:

Background: Mesenchymal stem cells (MSCs) as pluripotent stem cells are able to migrate toward tumor cells and inflammation due to uPA and uPAR expression in these cells and tumor cells. Platelet releasate (PR) contains different growth factors can be used for FBS replacement in cell culture medium for in vitro expansion of MSCs in cell therapy. In this study Mesenchymal cells were exposed to PR and the expression of uPA and uPAR gene and protein was investigated.

Methods and Materials: Bone marrow derived MSCs were characterized by flow cytometric analysis of specific surface markers and osteoblastic differentiation ability. These cells were cultured in the complete medium supplemented with (10% FBS), (10% PR) and (5% FBS - 5% PR) for 7 days. The PR was prepared by adding thrombin to random donor platelets derived PRP. Expression of uPA and uPAR genes was examined by Real time PCR. uPA and uPAR protein expression was assessed by Western blot.

Results: The results showed that uPA and uPAR gene expression was increased significantly in two culture conditions including (5% FBS - 5% PR) and (10% PR) compared to 10% FBS ($P<0.01$). The highest uPA and uPAR gene expression was observed in (10% PR) and there were significant differences between (10% PR) and (5%FBS -5%releasate) ($P<0.01$). uPA and uPAR protein expression was detected in (5% FBS - 5 % releasate) and (10% releasate) culture condition.

Conclusion: Our results was stated that use of PR in MSCs culture medium enhance expression of uPA and uPAR molecules on MSCs, and probably can be increased tropism ability to tumor cells.

Key Words: Mesenchymal stem cells, uPA, uPAR, Tropism

Introduction

Several studies suggest that Mesenchymal cells can be used to cell-based different therapeutic manners. One of the most application of MSCs are in the treatment of incurable diseases like cancers. As it well known despite much progress in the management of cancer, it is still a major public health problem on (1-6). On the other hand other tissue regeneration as a new point of view in the medicine could be achieved mediated by MSCs. The ability of mesenchymal cells in migrating to damaged tissues and tropism toward tumor cells was the reasons to be candidate for cancer cell therapy (7-9). This has led to the efforts in the targeting this migration and destruction of tumors and inhibition of inflammation (10, 11). Migration of mesenchymal cells toward the tumor is led to make a

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microenvironment which results in protection of solid tumors in a safe three-dimensional area. (11). These cells can be used as vehicles for inhibitory factors affecting the survival of tumor cells, oncolytic genes and cytotoxic drugs in treatment of different malignancies(12), nano particles marked MSCs also could be used in tumor diagnosis using modern methods of radiology . These methods could be used in diagnosis and treatment of multiform glioblastoma, prostatic, melanoma, liver tumors and other malignancies (13). Tropism or patotropism is the ability of the specific migration of progenitor/stem cells from bone marrow to sites of inflammation or tumor by the aid of releasing inflammatory-like substances by tumor cells (14). Incubation of bone marrow isolated mesenchymal cells with cytokines and growth factors such as PDGF, IGF-1.SDF-1 increase attracting the MSCs toward tumor and inflammation site (13). Migration of these cells also is controlled by a large number of tyrosine kinase receptors family and cytokines: CXCR4- SDF-1HIF-1, SCF-Ckit, and HGF-cMet (15). Along with recognizing mesenchymal stem cells as a transportation tool of therapeutic agents toward the tumor site, signaling pathways which mediate the migration of mesenchymal cells were introduced. uPA (Urokinase plasminogen activator) and its receptor uPAR(CD 87) over expressed in malignancies such as solid tumors of brain, prostate, lung and breast. This may result an increase in the migration of cells to the tumor site, and the absence of uPA may cause lack of cell migration. On the other hand chemo attractive cytokines such as IL6, IL8 and MCP-1 have an important role in the uPA-uPAR reaction (15). Platlet releasate (PR) as a replacement for FBS contain molecules and cytokines that are needed for mesenchymal cell growth in the cell culture medium (16). Mesenchymal stem cells are more potent in colony forming with the presence of 5% PR in comparison with FCS. (16,17) Similar to FCS, effect of PR on mesenchymal cells proliferation, phenotype and differentiation capability was proven (18).

The hypothesis of effectiveness of PR was strengthened by the fact that growth factors such as IGF 1&2, VEGF, HGF/SF, CSF-1, LPA, bFGF, vasopressin and thrombin are able to increase the expression of uPA. By attaching these factors to the ligands, PLC, CD42, Raf, Rac, Rho, Ras, PKC, MEK 1& 2 will be activated and transcription of the uPA gene would be increased. Therefore PRs has a fundamental role in increase of expression of uPA due to the growth factors such as PDGF, IGF 1&2 and VEGF (19). In this study Mesenchymal cells were exposed to PR and the over expression of uPA and uPAR genes and up regulation of uPA and uPAR proteins was investigated.

Material and Methods

PR Preparation

To prepare thrombin, 10 mL citrated blood was centrifuged at 1800g for 10 minutes. Then calcium gluconate (100 mg/ml) was added to platelet free plasma and incubated for 30 min at 37°C. After clotting, samples were centrifuged 1800g in 10 minutes and the supernatant containing thrombin was collected, filtered with a 0·22-µm pore filter, divided into aliquots and frozen at –80°C for further use. For preparation of platelet rich plasma (PRP), platelet concentration was centrifuged for 10 minutes at 1800g for removing plasma. The platelet precipitate was suspended in PBS buffer resulting in a platelet number of 1×10^6/ml.Then 2 ml thrombin and 2.5 ml calcium gluconate was added to PRP and incubated at 37°C for 30 min. After 10 minutes centrifugation at 1800g for 10 minutes, the supernatant containing growth factors released from platelets was removed, divided into aliquots and frozen at –80°C for further use.

Bone marrow mesenchymal stem cell isolation

5 ml of heparinized bone marrow aspirate samples from healthy consenting donors was collected in heparin tube and mixed with 5 ml of DMEM (Sigma, USA). The mononuclear cell layer was isolated by Ficoll-Hypaque, washed in PBS, resuspended in growth medium containing low glucose DMEM supplemented with 10% FBS(Stem cell Technology, USA), 100 µg/ml streptomycin, and 100 U/ml penicillin (Iran-CinnaGen), and plated in 25-cm^2 plastic cell culture flasks. The cell cultures were maintained at 37°C in a humidified 5% CO2 incubator. After 48 hours the supernatant was discharged for removing hematopoietic cells and the medium was replaced every 3 days for 14 days.

Immunophenotyping analysis of MSCs

MSCs were characterized by flow cytometric analysis using monoclonal antibodies against CD105, CD44, CD166, CD90, CD34, and CD45. Trypsinated MSCs resuspended in phosphate buffer with FITC- or PE-coupled antibodies against CD105, CD90, CD166, CD45 and CD44 or the mouse IgG1 isotype negative control(Dako, Denmark) for 30 min at 4°C. The cells were washed with PBS-2%BSA and examined using a flow cytometry (Partec, Germany) and Flowmax software.

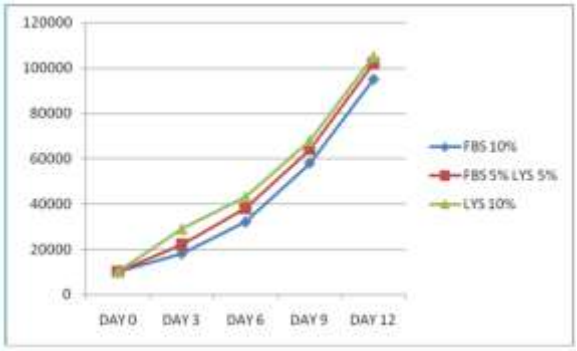
Osteoblastic differentiation assay

Osteogenic differentiation was performed on bone marrow derived MSC. For this purpose, $5x10^4$cells were seeded in collagen / Vitro nectin coated 24-well plates. After 100% confluences DMEM enriched with FBS were replaced with differentiating medium containing 100 µm l-ascorbic acid-2-phosphate and 1m β-glycerophosphate with 1mm dexamethasone (Chemicon, USA). On day 14, cells were fixed in 70% ethanol for 1 h at 4 °C and stained for 15 min with alizarin red-S (Sigma) at room temperature (RT). Alkaline phosphatase staining was performed with alkaline phosphtase kit (Sigma-Aldrich). Differentiated cells in the 24-well plate were fixed with acetone and Fast Blue RR salt in Naphthol AS-MX Alkaline solution was use for staining. Mayer's Hematoxylin was used as counterstaining.

Effect of PR on MSCs

$1×10^5$ MSCs were seeded in 6-well plates and cultivated in low glucose DMEM medium supplemented with 10%FBS or 5%FBS & 5%releasate or releasate 10% for 7 days at 37°C under 5% CO2 humidified air. The media was replaced every other day. In order to evaluate cell proliferation after adding trypsin and removing cells attached to the surface of flask, the medium including suspending cells were transferred to falcon tube. Then it was centrifuged for 1o min at 450 g. supernatant was removed and cells were suspend in 1 ml of medium. Then with the use of neubauer chamber cells were count in white cell area. With the help of followed formula cell count was calculated.

The exact date are shown on graph1



graph1: Assessment of Mesenchymal stem cell proliferation in three different medium.

Expression analysis of genes via quantitative real-time PCR

RNA was extracted from expanded cells in all the three culture conditions at day 7 of culture using Trizol reagent (Roche,Germany). They were immediately frozen and kept at -70°C until use. Reverse transcription was performed on 1 µg total RNA, with AccuPower CycleScript RT PreMix reverse transcription Kit (Bioneer, USA). The reaction was carried out in 12 cycles of 22°C for 30s, 45°C for 4min; 55°C for 30s, and 1 cycle of 95°C for 5 min. Products were analyzed in 1.5% agarose gel under UV light. Quantitative RT-PCR was performed with the LightCycler technology, using 2 µL cDNA in 25 µL reaction volume with 0.4 µM of each primer and 12.5 µL of 2x Fast Start DNA Master SYBR Green I (Roche Molecular Biochemicals, Germany). Thermal cycling was initiated at 95°C for 5 min followed by 40 cycles of PCR (95°C, 30 s; 62°C, 30s; 72°C, 20s). GAPDH was used as an endogenous control. Fold change ratio was calculated via $2^{\wedge\wedge Ct}$ method. The Reverse and forward primers designed by GeneRunner software 3.05 version are as follows:

uPA-R: 5- TAC ATC GAG GGC AGG CAG ATG GT- 3

uPA -F: 5- GCT GAC ACG CTT GCT CAC CAC A- 3

uPAR-R: 5- TGC ATT CGA GGT AAC GGC TTC GG- 3

uPAR-F: 5- ACC CTG AGC TAT CGG ACT GGC TT- 3

Protein expression analysis by Western Blot

Cultured cells were lysed in RIPA buffer directly on culture dishes. Releasates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The protein was transferred onto a poly vinylidene fluoride membrane (Roche Diagnostics). The membranes were developed with primary antibodies against uPA and uPAR proteins (1:1000 Mouse monoclonal IgG2a, R&D, USA). After washing, the membrane was further incubated with HRP-conjugated anti-mouse secondary antibody (1:3000; Santa Cruz Biotechnology, CA, USA) at room temperature for 2 h. Then, bound antibody was revealed using 3, 3′-diaminobenzidine as the substrate (DAB; 0.5 mg/mL). Finally, the membranes were dried and then scanned using photo scanner. Molecular mass was estimated by reference to standard proteins (Fermentas, GmbH, St. Leon-Rot, Germany).

Results

Mesenchymal stromal cells Characterization

MSCs Immunophenotyping

MSCs isolated from bone marrow were characterized by flow cytometric analysis of specific surface antigens. MSCs were found to be positive for the following adhesion molecules: CD44 (99.5%), CD166 (98.8%), CD105 (93%) and CD90 (96.8%) which together were considered as markers for MSCs. As shown in Fig. 1, the MSCs were negative for haematopoietic lineage markers, namely, CD34, and CD45. (Figure 1)

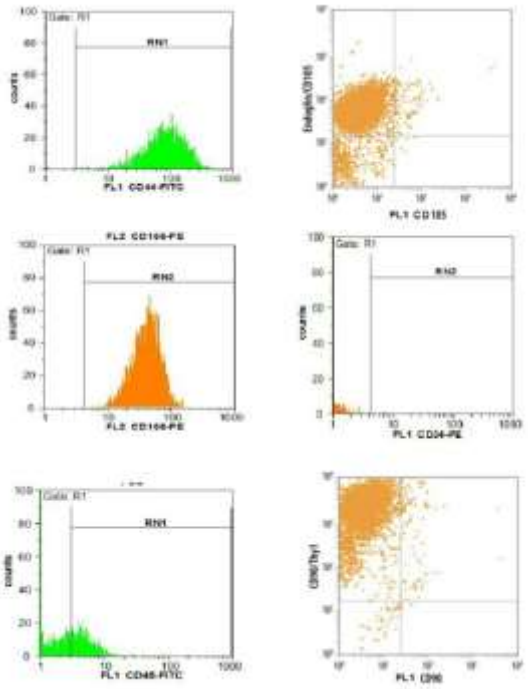


Figure 1: Evaluation of the surface markers expression on bone marrow mesenchymal stem cells using flow cytometry
A: CD 105, 93 %
B: CD 166, 98.8 %
C: CD44, 99.5 %:
D: CD 90, 96.8 %
E: CD 45, 3.8%
F: CD 34, 2.8%

Osteogenic differentiation assay

Osteogenic differentiation potential of bone marrow derived MSCs was assyed by alizarian red staining and evaluation of alkaline phosphatase activity. Both Alizarin red staining and alkaline Phosphatase activity showed a positive reaction after 14 days.

Expression of uPA and uPAR gene after releasate/FBS treatment

Expression of uPA and uPAR gene in mesenchymal stem cells in all the three culture conditions was evaluated by real time RT-PCR after 7 days. The results showed that uPA gene expression was increased significantly in two culture conditions including 5% FBS - 5% releasate (1.7±0.3 times) and 10% releasate (3.1± 0.5 times) compared to 10% FBS medium ($P<0.01$). (Figure 2)

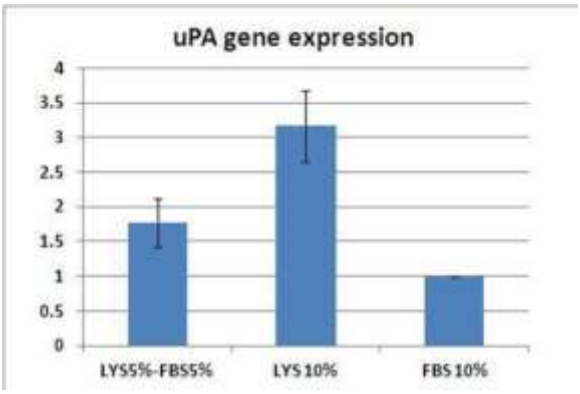


Figure 2: Expression of uPA after releasate/FBS treatment

uPAR gene expression was also increased significantly in two culture conditions including 5% FBS- 5% releasate (1.3±0.3 times) and 10% releasate (2.1±0.6 times) compared to 10% FBS medium(P<0.01).(Figure 3)

The highest uPA and uPAR gene expression was observed in 10% releasate Medium and there were significant differences between 10% releasate and 5% FBS-5% releasate media. (P<0.01)

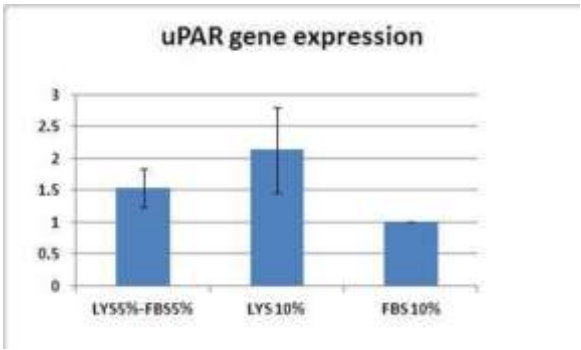


Figure 3: Expression of uPAR after releasate/FBS treatment

Protein expression of uPA and uPAR after releasate/FBS treatment

Expression of uPA and uPAR protein in mesenchymal stem cells in all the three culture conditions was evaluated by Western-blot analysis. The results showed that a single band of approximately 54 kDa for uPA and 50 kDa for uPAR protein in extracted samples of all the three culture conditions. (Figure 4)

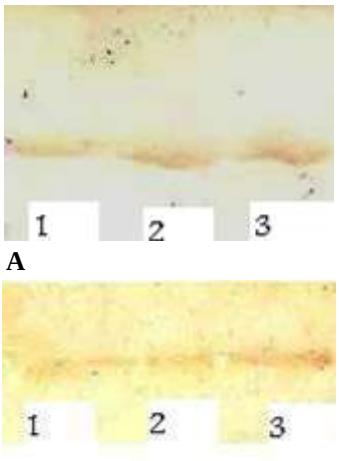


Figure 4: uPA and uPAR protein expression after releasate/FBS treatment

A: uPA protein bands B: uPAR protein bands

In the three culture condition:

1-10% FBS

2-5% FBS - 5% PR

3- 10% PR

Discussion

Pooled human AB serum: serum with AB group derived from several people. The ability of mesenchymal cells in migrating to damaged tissues and tropism toward tumor cells was the reasons to be candidate for cancer cell therapy (10,11). As uPA and its receptor uPAR over expressed on tumor cells and mesenchymal stem cells in malignancies, they were known as one of the most interfering agents in tropism. Furthermore chemo attractive cytokines have an important role in the uPA-uPAR interaction (15). Mesenchymal stem cells are more potent in colony forming with the presence of 5% PR in comparison with FCS (16). Similar to FCS effects on mesenchymal cells phenotype, differentiation and increasing proliferation (16,17), PR can be substituted in the cell culture medium and because of great content of growth factors such as VEGF, HGF/SF, CSF-1, LPA, bFGF, and thrombin it can be act as an enhancer of uPA expression (18,19).

In this study MSCs were cultured in the complete medium supplemented with (FBS 10%), (PR 10%) and (FBS & PR 5%) for 7 days. MSCs proliferation in LYS 10% was more than two others and also the number of cells in 5% FBS- 5% Lys compared to control cells treated with 10% FBS was higher

proliferative. Bernardo et al in 2007 evaluated biological properties of MSCs expanded in medium supplemented with platelet-releasate (PL) for cell-therapy approaches. Their data supported the immunological functional plasticity of MSCs and suggested that MSCs-PL can be used as an alternative to MSCs-FCS (18). Kocaoemer et al in 2007 demonstrated higher proliferative effect of pooled human AB serum and thrombin-activated platelet-rich plasma on adipose tissue MSCs than does FCS. They showed that pooled human AB serum and thrombin-activated platelet-rich plasma are alternatives to FCS for adipose tissue MSCs (20). Doucet et al in 2005 demonstrated that PL-containing medium is enriched by growth factors (platelet-derived growth factors (PDGFs), basic fibroblast growth factor (bFGF), transforming growth factor (TGF-beta), insulin-like growth factor-1 (IGF-1) ...) and showed that PL is able to promote MSC expansion, to decrease the time required to reach confluence, and to increase CFU-F size, as compared to the FCS medium (21).

Our findings showed that uPA and uPAR gene expression was increased significantly in two culture conditions including 5%FBS - 5 % releasate and 10% releasate compared to 10%FBS medium. The highest uPA and uPAR gene expression was observed in 10%releasate Medium and there were significant differences between 10%releasate and 5% FBS -5% releasate media ($P<0.01$). The above results were confirmed with uPA and uPAR protein detection in both (5% FBS - 5% releasate) and (10% releasate) culture condition. Our study did not examine the uPA and uPAR over expression reasons. However, this increase is likely due to platelet growth factors release. Chang et al in 2008 examined the effect of IGF, one of the factors released from platelets, on uPA gene expression in myocardial cells. They resulted in significant time-dependent increases in tissue-type plasminogen activator (tPA) and uPA (22). Pulukuri et al in 2010 showed over expression of uPA in CB-MSCs induced migration capacity toward human cancer cells in vitro. In addition, theit results showed that uPA-uPAR knockdown in prostate cancer cells significantly inhibited tumor-specific migration of uPA-over expressing MSCs. These results have significant implications for the development of MSC-mediated, tumor-selective gene therapies (23).

Conclusion

Our results stated that use of PR in MSCs culture medium enhance expression of uPA and uPAR molecules on MSCs, and probably can be increased tropism ability to tumor cells.

This founding enhance the utility of such a convenient material like RP which can be less harmful. On the other hand the exact detail of how we can use MSC as a carrier remains controversial.it must be time consuming and expensive.

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